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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/14, 45/06	A1	(11) International Publication Number: WO 92/20369
		(43) International Publication Date: 26 November 1992 (26.11.92)

(21) International Application Number: PCT/US92/04068

(22) International Filing Date: 14 May 1992 (14.05.92)

(30) Priority data:
699,769 14 May 1991 (14.05.91) US

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(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BG, BR, CA, CH, CH (European patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC (European patent), MG, MN, MW, NL, NL (European patent), NO, PL, RO, RU, SD, SE, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF HEMOGLOBIN IN A METHOD FOR THE TREATMENT OF TUMORS WITH CHEMOTHERAPEUTIC AGENTS

(57) Abstract

A method is disclosed treating a tumor in a host by administering an ultrapurified polymerized hemoglobin solution to the host and also administering a chemotherapeutic agent to the host. In a particularly preferred embodiment, the hemoglobin is bovine hemoglobin.

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USE OF HAEMOGLOBIN IN A METHOD FOR THE TREATMENT OF TUMOURS WITH CHEMOTHERAPETIC AGENTS

Description

Background of the Invention

Solid tumor masses in cancer patients have been found to be heterogeneous in oxygenation and to contain regions of hypoxia. See Vaupel. P., "Oxygenation of Human Tumors", Strahlenther. Onkol. 166:377-386 (1990); and Adams, G.E., The Clinical Relevance of Tumour Hypoxia, 26(4):420-421 (1990). Recent studies in human tumors with oxygen electrodes have reaffirmed the occurrence of significant hypoxic areas within human tumors. Vaupel, P. ibid; Kallinowski, F. et al., "Tumor Tissue Oxygenation as Evaluation by Computerized-pO_2-Histography", Int. J. Radiat. Oncol. Biol. Phys. 19:953-961 (1990); and Gatenby, R.A. et al., "Oxygen Distribution in Squamous Cell Carcinoma Metastases and Its Relationship to Outcome of Radiation Therapy", Int. J. Radiat. Oncol. Biol. Phys. 14:831-838 (1988). Preclinical studies, both in vitro and in vivo, have established that hypoxia protects tumor cells from the cytoxic actions of radiation and chemotherapeutic agents and thereby may be a significant factor in therapeutic resistance. Adams, G.E. ibid; Sartorelli, A.C., "Therapeutic Attack of Hypoxic Cells of Solid Tumors: Presidential Address", Cancer Res. 48:775-778 (1988); Teicher, B.A. et al., "Classification of Antineoplastic Agents by Their Selective Toxicitities Toward Oxygenated and Hypoxic

Tumor Cells", Cancer Res. 41:73-81 (1981); and Teicher, B.A. et al., "Classification of Antineoplastic Treatment by Their Differential Toxicity Toward Putative Oxygenated and Hypoxic Tumor Subpopulations in vivo in the FSaIIC Murine Fibrosarcoma", Cancer Res. 503339-3344 (1990).

Increased delivery of oxygen from the lungs can be a useful way of improving the oxygenation of solid tumor masses by altering the gradient of oxygen as it is absorbed from the vasculature and distributed into the tissue. Because of this, one strategy which has been attempted to overcome the problem of hypoxia in treating tumors involves the use of perfluorocarbon emulsions with oxygen or carbogen (95% oxygen/5% carbon dioxide) breathing. Holden, S.A. et al., "Addition of a Hypoxic Cell Selective Cytotoxic Agent (mitomycin C or porfiromycin) to Treatment with Fluosol-D® /Carbogen/Radiation", Radiother. Oncol. 18:59-70 (1990); Teicher, B.A. et al., "The Effect of Fluosol-DA and Oxygenation Status on the Activity of Cyclophosphamide In Vivo" Cancer Chemother. Pharmacol. 21:286-291 (1988); Martin, D.F. et al., "Enhancement of Tumor Radiation Response by the Combination of a Perfluorochemical Emulsion and Hyperb aric Oxygen", Int. J. Radiat. Oncol. Biol. Phys. 13: 747-751 (1987); Teicher, B.A. and C.M. Rose, Perfluorochemical Emulsion Can Increase Tumor Radiosensitivity" Science 223:934-936 (1984); and Teicher, B.A. et al., "Optimization of

Perfluorochmical Levels with Radiation Therapy" Cancer Res. 49:2693-2697 (1989). In preclinical solid tumor models, the use of perfluorocarbon emulsions with carbogen or oxygen breathing in conjunction with radiation therapy has produced positive results. Teicher, B.A. and C. M. Rose, ibid; Teicher, B.A. et al., ibid; Teicher, B.A. and C. M. Rose, "Oxygen-Carrying Perfluorochemical Emulsion as an Adjuvant to Radiation Therapy in Mice", Cancer Res. 44:4285-4288 (1984); Teicher, B.A. and C.M. Rose, "Effect of Dose and Scheduling on Growth Delay of the Lewis Lung Carcinoma Produced by the Perfluorochemical Emulsion, Fluosol-DA", Int. J. Radiat. Oncol. Biol. Phys. 12:1311-1313 (1986); Teicher, B.A., et al., "Influence of Scheduling Dose and Volume of Administration of the Perfluorochemical Emulsion Therox® on Tumor Response to Radiation Therapy", Int. J. Radiat. Oncol. Biol. Phys., in press (1991); Teicher, B.A. et al., "Effect of Fluosol®-DA on the Response of Intracranial 9L Tumors to X-rays and BCNU", Int. J. Radiat. Oncol. Biol. Phys. 15:1187-1192 (1988); Lee, I. et al., "Effects of Fluosol-DA and Carbogen on the Radioresponse of SCK Tumors and Skin of A/J Mice". Radiat. Res. 112:173-182 (1987); Martin, D.F. et al., "Effect of a Perfluorochemical Emulsion on the Radiation Response of BA 1112 Rhabdomysarcomas", Radiat. Res. 112:45-53 (1987); Mouler, J.E. et al., "Applicability of Animal Tumor Data to Cancer Therapy in Humans".

Int. J. Radiat. Oncol. Biol. Phys. 14:913-927 (1988); Moulder, J.E. and B.L. Fish, "Tumor Sensitization by the Intermittent use of Perfluorochemical Emulsions and Carbogen Breathing in Fractionated Radiotherapy", In: E.M. Fielden, J.F. Fowler, J.H. Hendry and D. Scott (eds.), Proceedings of the 8th International Congress of Radiation Research, Vol. 1, p. 299, London: Taylor and Francis, Inc. (1987); Rockwell, S. et al., "Reactions of Tumors and Normal Tissues in Mice to Irradiation in the Presence and Absence of a Peurofluorochemical Emulsion" Int. Radiat. Oncol. Biol. Phys. 112:1315-1318 (1986); Song, C.W. et al., "Increase in pO_2 and Radiosensititivty of Tumors by Fluosol®-DA (20%) and Carbogen", Cancer Res. 47:442-446 (1987); and Zhang, W.L. et al., "Enhancement of Tumor Response to Radiation by Fluosol-DA", Int. J. Radiat. Oncol. Biol. Phys. 10:172-175 (1984).

Further, some initial clinical trials of the perfluorochemical emulsion, Fluosol®-DA and oxygen breathing with radiation therapy have been carried out and some are still underway. Rose, C.M. et al., "A Clinical Trial of Fluosol®-DA 20% in Advanced Squamous Cell Carcinoma of the Head and Neck", Int. J. Radiat. Oncol. Biol. Phys. 12:1325-1327 (1986); Lustig, R. et al., "Phase I-II Study of Fluosol-DA and 100% Oxygen Breathing as an Adjuvant to Radiation in the Treatment of Advanced Squamous Cell Tumors of the Head and Neck", Int. J. Radiat. Oncol. Biol. Phys. 16:1587-1594 (1989); Lustig, R. et al., "Phase

I/II Study of Fluosol and 100% Oxygen Breathing as an Adjuvant to Radiation in the Treatment of Unresectable Non-Small Cell Carcinoma of the Lung", Int. J. Radiat. Oncol. Biol. Phys. 17SL:202 (1989); and Evans, R.G. et al., "A Phase I-II Study of the Use of Fluosol®-DA 20% as an Adjutant of Radiation Therapy in the Treatment of Primary High-Grade Brain Tumors", Int. J. Radiat. Oncol. Biol. Phys. 1721:175 (1989).

The effect of perfluorocarbon emulsions in carbogen or oxygen breathing with certain chemotherapeutic agents has also been studied in preclinical solid tumor models. Teicher, B.A. et al., "Classification of Antineoplastic Treatments by Their Differential Toxicity Toward Putative Oxygenated and Hypoxic Tumor Subpopulations in vivo in the FSaIIC Murine Fibrosarcoma", Cancer Res. 50:3339-3344 (1990); Holden, S.A. et al., "Addition of a Hypoxic Cell Selective Cytotoxic Agent (Mitomycin C or Porfiromycin) to Treatment with Fluosol-DA®/Carbogen/Radiation", Radiother. Oncol. 18:59-70 (1990); Teicher, B.A. et al., "The Effect of Fluosol-DA and Oxygenation Status on the Activity of Cyclophosphamide in vivo" Cancer Chemother. Pharmocol. 21:286-291 (1988); Teicher, B.A. et al., "Approaches to Defining the Mechanism of Fluosol-DA 20%/Carbogen Enhancement of Melphalan Antitumor Activity", Cancer Res. 47:513-518 (1987); Teicher, B.A. et al., "Differential Enhancement of Melphalan

Cytotoxicity in Tumor and Normal t+Tissue by Fluosol-DA and Oxygen Breathing", Int. J. Cancer 36:585-589 (1985); Teicher, B.A. et al., "Effects of Various Oxygenation Conditions on the Enhancement by Fluosol-DA of Melphalan Antitumor Activity", Cancer Res. 47:5036-5041 (1987); Teicher, B.A. and S.A. Holden, "A Survey of the Effect of Adding Fluosol-DA 20%/O_2 to Treatment with Various Chemotherapeutic Agents", Cancer Treat. Rep. 71:173-177 (1987); Teicher, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol-DA on Cancer Chemotherapeutic Agents", Biomat., Art. Cells and Art. Organs 16:533-546 (1988); Teicher, B.A. et al., "Effect of Oxygen on the Cytotoxicity of Antitumor Activity of Etoposide", J. Natl. Cancer Inst. 75:1129-1133 (1985); Teicher, B.A. et al., "Effect of Fluosol-DA/O_2 on Tumor Cell and Bone Marrow Cytotoxicity of Nitrosoureas in Mice Bearing FSaII Fibrosarcoma", Int. J. Cancer 38:285-288 (1986); Teicher, B.A. et al., "Effect of Fluosol-DA/O_2 on the Antitumor Activity and Pulmonary Toxicity of Bleomycin", Cancer Chemother. Pharmacol. 18:213-218 (1986); Teicher, B.A. et al., "Effects of Fluosol®-DA and Oxygen Breathing on Adriamycin Antitumor Activity and Cardiac Toxicity in Mice", Cancer 61:2196-2201 (1988); Teicher, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol®-DA on the Cytotoxicity and Antitumor Activity of Bleomycin", J. Natl. Cancer Inst. 80:599-603 (1988); Teicher, B.A. et al., "Effect of Fluosol-

DA/Carbogen on Etoposide/Alkylaing Agents Antitumor Activity", Cancer Chemother. Pharmacol. 21 281-285 (1988); Martin, D.F. et al., "Potentiation of Rat Brain Tumor Therapy by Fluosol and Carbogen", NCI Monogr. 6:119-122 (1988); and Kim, G.E. and C.W. Song, "The Influence of Fluosol-DA and Carbogen Breathing on the Antitumor Effects of Cyclophosphamide In Vivo", Cancer Chemother. Pharmacol. 25:99-102 (1989). With many chemotherapeutic agents, very positive therapeutic results h ave been obtained and several initial clinical trials have been carried out with Fluosol-DA and oxygen breathing with single anticancer drugs. See Gruber, M. et al., "Phase I/II Study of Fluosol®/O_2 in Combination with BCNU in Malignant Glioma", Proc. Amer. Assoc. Cancer Res. 31:190 (March 1990); Carewal, H. et al., "Fluosol®/Oxygen in Combination with Cyclophosphamide in Advanced Non-Small Cell Lung Carcinoma (NSCLC): Phase I Results", Proc. Amer. Assoc. Cancer Res. 30:271 (March 1989); and Meyers, F. et al., "Phase I/II Study of Fluosol®/Oxygen in Combination with Weekly 5-Fluororacil (5FU) in Metastatic Colorectal Carcinoma", Proc. Amer. Assoc. Cancer Res. 30:256 (March 1989).

Despite the initial success with the use of perfluorocarbon emulsions and carbogen or oxygen breathing in conjunction with ionizing radiation, these techniques have not proven entirely

satisfactory. For example, perfluorocarbons have very limited oxygen-transport capability at ambient oxygen pressures. Blood delivers approximately 6% (v/v) oxygen to tissues at ambient pressures, whereas, at these same pressures, perfluorocarbon emulsions can only delivery about 2% (v/v).

Summary of the Invention

This invention relates to a method for treating a tumor in a host, including a human being, with ionizing radiation. In this method, an ultrapurified polymerized hemoglobin solution (UPHS) is administered to the host in an amount which significantly increases the antitumor effect of the radiation. In addition, the use of the hemoglobin solution, in contrast to the use of perfluorocarbon emulsions, has certain advantages. Hemoglobin is able to chelate and deliver oxygen under air-breathing conditions. Polymerized hemoglobins have a longer circulating half-life than many of the perfluorocarbon emulsions and, therefore, have a longer functional period post-administration. The acidic environments in tumors increase the off-loading of oxygen and, therefore, the oxygen delivery from hemoglobin, as should temperature elevation (i.e., clinical hyperthermia). Hemoglobin solutions also have less retention in normal tissues, which is a concern with many perfluorocarbon preparations.

Brief Description of the Figure

Figure 1 is a plot of the surviving fraction of FSaIIC tumor cells and bone marrow granulocyte-macrophase colony forming units (CFU-GM) versus dose of Melphalan (MEL) administered to mice alone or with a single dose of ultrapurified polymerized bovine hemoglovin solution (UPBHS) administered immediately prior to MEL injection.

Figure 2 is a plot of the surviving fraction of FAsIIC tumor cells and bone marrow CFU-GM versus dose of Cyclophosphamide (CTX) administered to mice alone or with a single dose of UPBHS immediately prior to CTX injection.

Figure 3 is a graph of pO_2 measurements made of 13672TB mammary carcinoma, using a histograph.

Figure 4 graphically illustrates that the ultrapurified polymerized bovine hemoglobin solution called Hemopure alters the oxygenation profile of the tumor.

Figure 5 is the pO_2 measurements made of 9L brain tumor using a histograph.

Figure 6 graphically illustrates that the ultrapurified polymerized bovine hemoglobin solution called Hemopure improvwews the oxygenation of the 9L tumor whether the animals were breathing air or carbogen.

Detailed Description of the Invention

This invention relates to a method for treating a tumor in a host. The host can be any species which develops solid tumors or masses of tumor cells containing oxygen heterogeneity. Examples of hosts include but are not limited to, reptiles, amphibians, avians and mammals, including human beings, as well as domestic animals such as dogs, cats, cows and horses.

Tumors treatable by this method include those in which oxygen heterogeniety, including regions of hypoxia, protect tumor cells against the cytotoxic action of chemotherapeutic agents. These are usually solid tumors, such as sarcomas, carcinomas, lymphomas, etc. However, in certain cases of dispersed tumor cells form which can produce regions of oxygen heterogeneity, as well.

As used herein, the terms "chemotherapeutic agent" is employed to include chemical and biological agents, including small molecules and larger molecules, such as peptides, proteins, lymphokines, antibodies, tumor necrosis factor, conjugates of antibodies with toxins, and other chemical or biological molecules which have an antitumor effect which is oxygen dependent.

There are a variety of known classes of small molecule antitumor chemotherapeutic agents. These include alkylating agents, such as Melphalan (MEL), Cyclophosphamide (CTX), cis-Diamminedichloroplatinum

(II) (CDDP) and N N'-bis(II-chloroethyl)-N-nitrosourea (BCNU). Another general class of antitumor chemotherapeutic agents are the antimetabolite, such as 6-Mercaptopurine, 5-fluorouracil, fluorodeoxyuridine, cytosine arabinoside, methotrexate and thioquinone. Antibiotics are another general class of antitumor chemotherapeutic agents including drugs such as actinomycin, daunorubicin, adriamycin and bleomycin. Still yet another class is the vinca alkaloids, including etoposide, vincristine and vinblastine.

Mixtures of more than one antitumor chemotherapeutic agent can, of course, be administered. Indeed, it is often preferred to use mixtures of antitumor agents to treat tumors, especially agents from the different classes of agents. For example, mixtures of methotrexate and a cis-platinum compound are often used to treat various tumors.

The chemotherapeutic agent can be administered to the host parenterally, for example, by subcutaneous, intravenous or intramuscular injection or by absorption through a bodily tissue, such as the digestive tract, the respiratory system or the skin. The form in which the antitumor agent is administered (e.g., capsule, tablet, solution, emulsion) will depend, at least in part, on the route by which it is administered.

The chemotherapeutic agent is administered in a therapeutically effective amount. This amount will be determined on an individual basis and will be based, at least in part, in consideration of the host's size, the specific tumor to be treated, the severity of the symptoms to be treated, the results sought, and other such considerations. An effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

In order to increase oxygen transport of the site of a tumor, an ultrapurified polymerized hemoglobin solution (UPBHS) is administered to the host. Although not essential, it is preferred to administer the UPHS prior to administration of the antitumor agent. Also, the hemoglobin solution is preferably administered intravenously so that it is taken into the bloodstream of the host immediately.

As mentioned above, it is preferably to administer UPHS prior to administration of the chemotherapeutic agent. The amount of time between the administration of the hemoglobin and chemotherapeutic agent will depend upon factors such as the amount of time it takes the hemoglobin solution to be fully incorporated into the circulatory system of the host, the lifetime of the hemoglobin solution, etc. Since polymerized bovine hemoglobin has been found to remain in the host's

blood stream for up to at least 48 hours, anytime during this period is sufficient.

Hemoglobin sufficient for the hemoglobin solutions can be derived from a wide variety of sources. These sources include human blood, such as outdated blood bank supplies. Additionally, the hemoglobin can be derived from a variety of mammalian sources such as horses, pigs, cows, sheep, etc.

In a preferred embodiment, the hemoglobin will be derived from a species in which the hemoglobin is chloride ion-dependent for oxygen transport rather than dependent upon 2,3-diphosphoglycerate (2,3-DPG) or other phosphate molecules. This is because 2,3-DPG, present in human red blood cells, is not available freely in the circulatory system of the host to effect oxygen uptake and release for hemoglobin solutions administered according to this invention. Thus, it is preferred to employ a hem globin which is chloride ion-dependent for oxygen transport, such as those hemoglobins derived from sheep, goats, cows and cats. See Bunn. J.F., "Differences in the Interaction of 2,3-Diposphoglycerate with Certain Mammalian Hemoglobins", Science 172:1049-50 (1971); Breepel, P.M. et al., "Interaction of Organic Phosphates with Bovine Hemoglobin -- I Oxylabile and Phosphate Labile Proton Binding", Pflugers Arch. 389:219-25 (1981); and Fronticelli, C. et al., "Solvent Regulation of Oxygen Affinity and Hemoglobin -- Sensitivity of

Bovine Hemo-Globin to Chloride Ions", J. Bio. Chem. 259:10841-4 (1984). Bovine hemoglobin is particularly preferred because of its proven ability to transport oxygen in human beings and other mammals, in a chloride ion-dependent way, and because of its low antigenicity in human beings when it has been ultrapurified.

In order to increase the useful life of hemoglobin in the circulation, it is polymerized or crosslinked by a variety of techniques. Crosslinking agents include dialdehydes, such as glyoxal, malonic dialdehyde, succinic dialdehyde, glutaraleehyde, adipaldeyde, 3-methylglutaraldehyde, propyladipaldehyde, phthalic dialdehyde, terephthaldehyde and malonic dialdehyde have been employed. See, in this regard, Bonsen et al., U.S. Patent Nos. 4,001,200; 4,001,401; and 4,053,590; Bonhard et al., U.S. 4,136,093 and U.S. Patent Nos. 4,336,248; the teachings of each of which are incorporated herein by reference.

The polymerized hemoglobin solution is ultrapurified by various filtration and chromatographic procedures which have been described heretofore in the art. An ultrapure hemoglobin solution, according to this invention, is a hemoglobin solution which is substantially free of stroma, endotoxin, other pyrogenic substances, phospholipids, immunoglobulins and cellular-contained enzymes.

A particularly preferred ultrapure polymerized hemoglobin solution is based upon bovine hemoglobin solution is based upon bovine hemoglobin. Such a bovine blood substitute has an endotoxin concentration of less than 0.5 endotoxin units/ml as measured by the LAL test; a phospholipid concentration of less than about 1 nanogram/milliliter and has a molecular weight distribution greater than 90% in the range of 68,000-500,000 daltons. This bovine hemoglobin solution also has an osmolarity measured by freezing point depression in the range of 180-320 milliosmols per liter; a hemoglobin content of 5-25 grams per deciliter; a met hemoglobin content of less than 20%; a p_{50} in the range of 18-36 mmHg; an intravascular half life of at least two days; a crosslinking profile on gel permeation chromatography of 50-70%.

Such ultrapurified polymerized bovine hemoglobin solution is made and sold by Biopure Corporation, Boston, MA under the trademark Hemopure. This and other ultrapurified hemoglobin solutions are described in International Patent Application PCT/US87/02967, published under WO88/03408, the teachings of which are hereby incorporated by reference.

Appropriate dosages of UPBHS can be determined by those skilled in the art using routine experimentation. The dose employed in the murine studies in the Examples herein was 12 ml/kg, which is

13%-15% of the estimated circulatory volume, or 1.32 g protein/kg. This dose corresponds to 840 ml as the comparative human dose or 17%-19% of estimated circulatory volume, and 2.4 g protein in a 70 kg person. Multiple doses of UPHS, for example one before each chemotherapy treatment, are, of course, useful with this invention and can be preferred in many cases.

The techniques for treating tumors described herein can be employed at normal body temperatures or at elevated body temperatures (hyperthermia).

Although not required, it is preferred to have the host breathe oxygen-enriched gas prior to and post administration of the ionizing radiation. This can be done by having the host breath oxygen-enriched air, 100% oxygen or carbogen (95% oxygen/5% C02), or in certain cases exposing the host to hyperbaric oxygen conditions.

This invention will now be further and more specifically described by the following examples.

Example I

Tumor Growth Delay with UPHBS

The FSaII fibrosarcoma (FSaIIC), adapted for growth in culture, was employed. See Rice, L. et al., "The Radiosensitivity of a Murine Fibrosarcoma as Measured by Three Cell Survival Assays", Br. J. Cancer 41:240-245 (1980). $2x10^6$ FSaIIC cells,

prepared from a brei of several stock tumors, were implanted intramuscularly into the legs of 8- to 10-week old male C3H/FeJ mice (The Jackson Laboratory, Bar Harbor, ME). When the tumors were approximately $100mm^3$ in volume, 0.3 ml (12 ml/kg; 1.32 gm protein/kg) of ultrapurified polymerized bovine hemoglobin solution (UPBHS) was injected via the tail vein. The UPBHS solution was obtained from Biopure Corporation, Boston, MA ans was a polymerized form of a highly purified bovine hemoglobin solution. It contained 11±2 gm/deciliter of bovine hemoglobin. P_{50} measurements of UPBHS in three assay systems and under conditions designed for testing human hemoglobin gave values of 17 mmHg to 23 mmHg. The hemoglobin content had a molecular weight of range from 68,000 to 500,000 (w/v). It contained sodium (145 mM/L), chloride (140mM/L) and potassium (4.0 mM/L) in a buffer solution (pH 7.8 ± 0.4). The circulating half life of this UPBHS was about 2.5 days. DeVenuto, F., "Evaluation of Human and Bovine Modified-Hemoglobin Solution as Oxygen-Carrying Fluid for Blood Volume Replacement", Biomat. Art. Cells, Art. Org. 16:77-82 (1988); and Winslow, R.M., "Optimal Hematologic Variables for Oxygen Transport Including P_{50}, Hemoglobin Cooperativity, Hematocrit, Acid-Base Atatus, and Cardiac Function", Biomat., Art. Cells and Art. Organs 16:149-172 (1988).

Immediately after the administration of UPBHS, 10 mg/kg Melphalan (MEL), 150 mg/kg Cyclophosphamide

(CTX), 10 mg/kg cis-diaminodichloroplatium(II) (CDDP) or 15 mg/kg N,N-bis(2-chloroethyl)-N-nitrosourea (BCNU) was administered by intraperitoneal unjection of 0.2 ml of phosphate buffered normal saline containing the drug. CTX and MEL were purchased as pure powders from Sigma Chemical Company, St. Louis, Missouri. CDDP was obtained as a gift from Bristol Meyers-Squibb Company, Wallingford, CT. BCNU (Carmustine) was purchased from the Dana-Farber Cancer Institute Pharmacy.

The mice were allowed to breath air or were placed in a circulating atmosphere of 95% 02/5% C02 (carbogen) for six hours and then removed to air. The progress of each tumor was measured three times weekly until it reached a volume of 500mm^3. Tumor growth delay was calculated as the days taken by each individual tumor to reach 500 mm^3 compared to the untreated controls. Each treatment group had seven animals and the experiment was repeated three times. Days of tumor growth delay are the mean $\pm$ SE for the treatment group compared to the control.

Data on the delay of tumor growth were analyzed with a BASIC program for the Apple II minicomputer. The program derives the best fit curve for each set of data, then calculated the median, mean and standard error of the mean for individual tumor volumes and the day on which each tumor reached 500 mm^3. Statistical comparisons were made with Dunn Multiple Comparisons Test.

The results of these tumor growth delay experiments are presented below in Table 1.

TUMOR GROWTH DELAYS, DAYS

Treatment Group	AA Alone	PBHS/ AIR	Carbogen	PBHS/ Carbogen
MEL (10mg/kg)	3.1±0.5	6.9±1.0	4.0±0.6	11.1±1.3
CTX (150mg/kg)	3.6±0.4	7.4±0.7	5.0±0.5	16.5±1.8
CDDP (10mg/kg)	7.4±0.8	9.6±1.1	7.6±0.3	14.1±1.6
BCNU (15mm/kg)	2.5±0.3	3.8±0.5	3.3±0.3	5.7±0.9

Neither PBS nor the PBS/carbogen alone had any effect on the growth of the FSaIIC fibrosarcoma. The addition of UPBHS to treatment with MEL resulted in a 2.2-fold increase in the tumor growth delay produced by MEL from about 3 days to about 7 days. Although carbogen breathing (6 hours) resulted in a small increase in tumor growth delay compared with MEL and air breathing, the combination of PBHS and carbogen produced a 3.6-fold increase in the tumor growth delay compared with MEL alone. The addition of UPBHS to treatment with a single dose of CTX resulted in a

2.1-fold increase in the tumor growth delay produced by CTX alone. Breathing a carbogen atmosphere for 6 hours post drug administration resulted in a small increase in the tumor growth delay produced by CTX; however, the combination of UPBHS and carbogen breathing was much more effective resulting in a 4.6-fold increase in tumor growth delay to about 16.5 days from 3.6 days for the drug alone.

The tumor growth delay produced by CDDP was less affected by the addition of PBHS to treatment with the drug than was either MEL or CTX. There was only a 1.3-fold increase in tumor growth delay with PBHS and CDDP compared with CDDP alone. Carbogen breathing for 6 hours following drug administration had no significant effect on the tumor growth delay produced by CDDP. The combination of PBHS and carbogen breathing was a more effective addition to treatment with C DDP and resulted in a 1.9-fold increase in tumor growth delay from 7.4 to 14.1 days.

The addition of PBHS to treatment with BCNU increased the tumor growth delay produced by BCNU by 1.5-fold. Although carbogen breathing for 6 hours post drug administration increased the tumor growth delay produced by BCNU to a small degree, a much larger enhancement in tumor growth delay was observed with PBHS and carbogen breathing in combination with BCNU. The combination of PBHS/BCNU and carbogen resulted in a tumor growth delay of about 5.7 days,

which was a 2.3-fold increase over the 2.5 days obtained with BCNU alone.

Example II

Effects of UPBHS on Tumor Cell Toxicity and Bone Marrow Toxicity of MEL

The procedures and materials of Example I were employed, except as noted. In this Example, tumors were allowed to grow to approximately 100 mm^3 in volume, which took about one week after tumor cell implantation. At this time 0.3 ml of UPBHS was injected via the tail vein. Immediately afterward, MEL was administered by intraperitoneal injection. The animals were then allowed to breathe air or were placed in a circulating atmosphere of carbogen for 6 hours and then removed to air. The mice were sacrificed 24 hours after treatment to allow for full expression of drug cytotoxicity and repair of potentially lethal damage. The tumors were excised under sterile conditions and single cell suspensions were prepared for a colony forming assay. See Teicher, B.A. et al., "Approaches to Defining the Mechanism of Fluosol-DA 20%/Carbogen Enhancement of Melphalan Antitumor Activity", Cancer Res.- 47:513-518 (1987); Teicher, B.A. et al., "Differential Enhancement of Melphalan Cytotoxicity in Tumor and Normal Tissue by Flusol-DA and Oxygen Breathing", Int. J. Cancer 36: 585-589 (1985); Teicher, B.A. et

al., "Effects of Various Oxygenation Conditions on the Enhancement by Fluosol-DA of Melphalan Antitumor Activity", Cancer Res. 47:5036-5041 (1987); Teicher, B.A. and S.A. Holden, "A Survey of the Effect of Adding Fluosol-DA 20%/O_2 to Treatment with Various Chemotherapeutic Agents", Cancer Treat. Rep. 71:173-177 (1987); Teicger, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol-DA on Cancer Chemotherapeutic Agents", Biomat., Art. Cells and Art. Organs 16:533-546 (1988). One week later, the plates were stained with crystal violet and colonies of more than 50 cells were counted. The untreated tumor cell suspensions had a plating efficiency of 8-12%.

Bone marrow toxicity was determined as follows. Bone marrow was taken from the same animals used for the tumor excision assays and colony forming assays were carried out in the same manner. Colonies of at least 50 cells were scored on an acculite colony counter (Fisher, Springfield, NJ). The results from three experiments, in which each group was measured in triplicate, were averaged.

The result for the tumor excision assays and bone marrow toxicity tests with MEL are plotted in Figure 1 wherein the surviving fraction ± SE of cells from the treated groups are compared to untreated controls.

MEL killed FSaIIC cells in a log-linear manner with increasing dose of MEL. With addition UPBHS to

treatment with MEl at a dose of 10 mg/kg, there was about a 10-fold increase in tumor cell killing compared with MEL along. At higher doses of MEl, the enhancement in tumor cell killing with the combination treatment disappeared indicating that whatever effect UPBHS had to effect tumor cell killing could also be accomplished by increased dosage of the alkylating agent. In the bone marrow CFU-GM, the addition of UPBHS to treatment with MEL produced a 3-fold increase in cell killing across the entire dosage range of MEL examined.

Example III
Effects of UPBHS on Tumor Cell Toxicity and Bone Marrow Toxicity of CTX

The procedures and materials of Example II were employed except that CTX was administered instead of MEL. The results are shown in Figure 2.

CTX killed increasing numbers of FSaIIC cells with increasing doses of the drug. The addition of PBHS to treatment with a dose of 100 mg/kg of CTX resulted in about a 20-fold increase in the killing of FSaIIC cells. Although the differential between tumor cell kill by CTX alone and CTX plus UPBHS decreased as the dose of CTX was increased, at the highest dose (500 mg/kg) of CTX examined, there was about 9-fold greater kill of FSaIIC tumor cells with the combined treatment. The addition of the UPBHS to

treatment with CTX resulted in a relatively small (2-3-fold) increase in the toxicity of the drug to bone marrow CFU/gm.

Example IV

Comparison Between Effects of UPHS and Perfluorocarbon Emulsion Employed in Combination with MEL

The procedures and materials of Example I were employed except that Fluosol-DA was substituted for UPHS. Fluosol-DA was obtained from Alpha Therapeutics Corporation and is an emulsion consisting of 25% (w/v) of the following perfluorocarbons: 7 parts perfluorodecalin; 3 parts per perfluorotripropyliamine; Pluronic F-68 (2.7%, w/v); yolk phospholipids (0.4% as emulsifiers; and glycerol (0.8%, w/v) as a cryoprotective agent. The annex solution (electrolyte/bicarbonate solution) furnished the preparation with physiological osmolarity.

The specific procedures employing Fluosol-DA have been described previously. See Teicher, B.A. et al., "Approaches to Defining the Mechanism of Fluosol-DA 20%/Carbogen Enhancement of Melphalan Antitumor Activity", Cancer Res. 47:513-518 (1987); Teicher, B.A. et al., "Differential Enhancement of Melphalan Cytotoxicity in Tumor and Normal t+Tissue by Fluosol-DA and Oxygen Breathing", Int. J. Cancer

36:585-589 (1985); and Teicher, B.A. et al., "Effects of Various Oxygenation Conditions on the Enhancement by Fluosol-DA of Melphalan Antitumor Activity", Cancer Res. 47:5036-5041 (1987).

When MEl (10 mg/kg) was administered to animals bearing the FSaIIC fibrosarcoma in combination with Fluosol-DA and air breathing a tumor growth delay of about 6.5 days was observed. If carbogen breathing for 1 hour post drug administration was added to therapy with Fluosol-DA and MEL a tumor growth delay of about 9.5 days resulted. Extending the carbogen breathing period to 6 hours did not alter the tumor growth delay produced by the MEl and Fluosol-DA combination (Teicher, B.A. et al., "Effect of Various Oxygenation Conditions and Fluosol-DA on Cancer Chemotherapeutic Agents", Biomat., Art. Cels and Art Organs 16:533-546 (1988)), however, preparation of the MEL in the Fluosol-DA as a vehicle resulted in a much enhanced tumor growth delay of about 29.5 days with carbogen breathing. The addition of PBHS to treatment with MEl as not quire as effective as combining MEL with Fluosol-DA and carbogen breathing resulting in a tumor growth delay of about 6.9 days. The combination of PBHS and carbogen breathing with MEL was more effective than the combination of Fluosol-DA and carbogen breathing with MEL producing a tumor growth delay of about 11.1 days compared with about 9.5 days.

Six hours of carbogen breathing are necessary to obtain a significant enhancement in the growth delay of the FSaIIC fibrosarcoma produced by CTX. Teicher, B.A. et al., "The Effect of Fluosol-DA and Oxygenation Status on the Activity of Cyclophosphamide In Vivo" Cancer Chemother. Pharmacol. 21:286-291 (1988). In the case of this drug the growth delay of the FSaIIC tumor with the treatment combination of CTX (150 mg/kg) with Fluosol-DA and carbogen breathing for 6 hours was about 12 days compared with about 3.6 days with CTX along. The 12 days of tumor growth delay obtained with this perfluorochemical emulsion/carbogen modulation of CTX was greater than the 7.4 days of tumor growth delay obtained with PBHS and air breathing with CTX but not as large as the 16.5 days of tumor growth delay obtained with the PBHS and carbogen breathing modulation of CTX.

Example V

The Eppendorf pO_2 histograph was used in the following experiment, this instrument allowed us to measure oxygen tension in tissues efficiently and reliably. We found the rat to be the preferred animal for these studies because it is easier to maintain their body temperature and respiration rate under anesthetic and because larger solid tumor masses can be grown in them.

For our initial studies with Hemopure we used the rat 13762TB mammary carcinoma and the rat 9L gliosarocoma, both implanted subcutaneously in the hind leg of Fisher 344 female rats (200-250 grams). The pO_2 measurements made in the 13672TB mammary carcinoma are depicted in Figure 1. Under normal air breathing conditions approximately 55% of the measured points (n=1640) are at values of <5 mm Hg and the average pO_2 in the tumor is 9.3 mm Hg. Upon administration of 8 ml/kg or 12 ml/kg iv of Hemopure, the average pO_2 in the tumors increases to about 22 mm Hg (n=500 and 180, respectively). Breathing carbogen (95% O_2, 5% CO_2) is effective in increasing the oxygenation of this tumor; however, the combination of Hemopure and carbogen breathing is most effective. When the dose of Hemopure was 8 ml/kg, the percentage of the tumor at a pO_2 <5 mmHg was reduced to about 16% and the average pO_2 was increased to about 37 mmHg. With the higher does of Hemopure of 12 ml/kg, the percentage of the tumor at pO_2 <5 mm Hg was about 3% and the average tumor pO_2 was about 50 mmHg. Figure 2 graphically demonstrates that Hemopure alters the oxygenation profile of the tumor primarily by increasing the oxygen tension in the more hypoxic 50 percentile of the tumor.

Our results thus far in the rat 9L gliosarocma are even more hopeful. In the 9L brain tumor model about 46% of the tumor has a po_2 of <5 mmHg and the average pO_2 is -7.4 mmHg under normal air breathing conditions (n=1862). (Figure 3). When Hemopure (12

ml/kg) was administered to the animals iv and normal air breathing maintained only about 13% of the tumor had a pO_2 of <5 mmHg and the average tumor pO_2 was about 18 mmHg. Carbogen is also effective in increasing the pO_2 of the 9L gliosarcoma. Under carbogen breathing conditions of 28% of the tumor had a pO_2 of <5 mmHg and the average tumor pO_2 was about 42 mmHg (n=1870). Administration of Hemopure (12 ml/kg) along with carbogen breathing further increased the oxygenation of the 9L tumor such that only about 1.5% of the tumor had a pO_2 <5 mmHg and the average tumor pO_2 was about 69 mmHg. Figure 4 graphically demonstrates that the administration of Hemopure improves the oxygenation of the more hypoxic 50 percentile of the 9L tumor whether the animals are breathing air or carbogen.

As shown on Table 2, the Hemopure preparation was more effective at enhancing the growth delay produced by the various chemotherapeutic agents. A dose of 12 ml/kg (0.3 ml/dose) appeared to be about optimal for use with the anticancer drugs.

TABLE 2. Growth delay of the FSalIC fibrosarcoma produced by various chemotherapeutic agents alone or in combination with Hemopure I or Hemopure II.

	TUMOR GROWTH DELAY, DAYS				
Treatment Group	Treatment Alone	+Hemo I (0.3 ml)	+Hemo II (0.3 ml)	+Hemo II (0.5 ml)	+Hemo I (1.0 ml)
Cyclophosphamide (3 x 150 mg/kg)	7.8	9.7	22.9	26.2	12.3
melphalan (10 mg/kg)	3.1	6.9	11.5	10.7	8.8
cisplatin (10 mg/kg)	4.4	5.9	6.9	7.1	5.7
carboplatin (3 x 50 mg/kg)	4.3	7.4	9.4	8.6	7.4
etoposide (3 x 15 mg/kg)	2.8	3.8	9.5	8.8	3.42
5-fluorouracil (5 x 40 mg/lg)	7.6			10.9	

CLAIMS

1. A method for treating a tumor in a host with a chemotherapeutic agent, comprising:
   a) administering to said host an ultrapurified polymerized hemoglobin solution in an amount sufficient to significantly increase the antitumor effect of said agent; and,
   b) administering to said host an effective amount of said chemotherapeutic agent.

2. A method of Claim 1 wherein said chemotherapeutic agent comprises an antitumor alkylating agent.

3. A method of Claim 1 wherein said hemoglobin comprises a hemoglobin which is dependent upon chloride ion concentration for oxygen transport.

4. A method of Claim 1 wherein said hemoglobin is bovine hemoglobin.

5. A method of Claim 4 wherein said chemotherapeutic agent comprises an antitumor alkylating agent.

6. A method of Claim 1 wherein said host is a mammal.

7. A method of Claim 6 wherein said mammalian host is a human being.

8. In a method of treating a tumor in a mammalian host with an antitumor alkylating agent:
The improvement of administering to said mammalian host, prior to treatment with said antitumor alkylating agent, an ultrapurified bovine hemoglobin solution in an amount sufficient to significantly increase the antitumor effect of said alkylating agent.

9. The improvement of Claim 8 wherein said mammalian host comprises a human being.

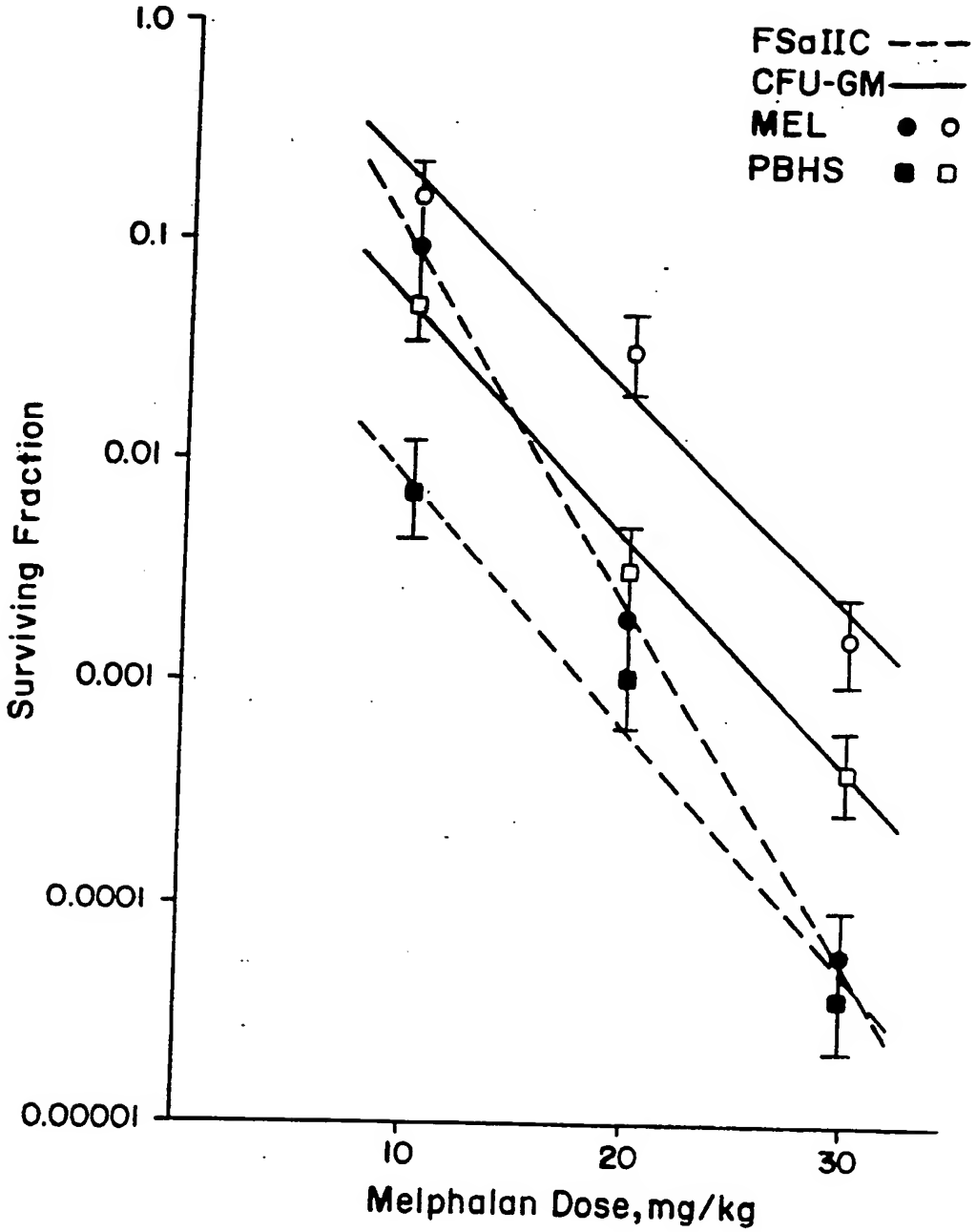
1.0
0.1
0.01
0.001
0.0001
0.00001
Surviving Fraction
10
20
30
Melphalan Dose, mg/kg
FSaIIC
CFU-GM
MEL
PBHS

FIG. 1

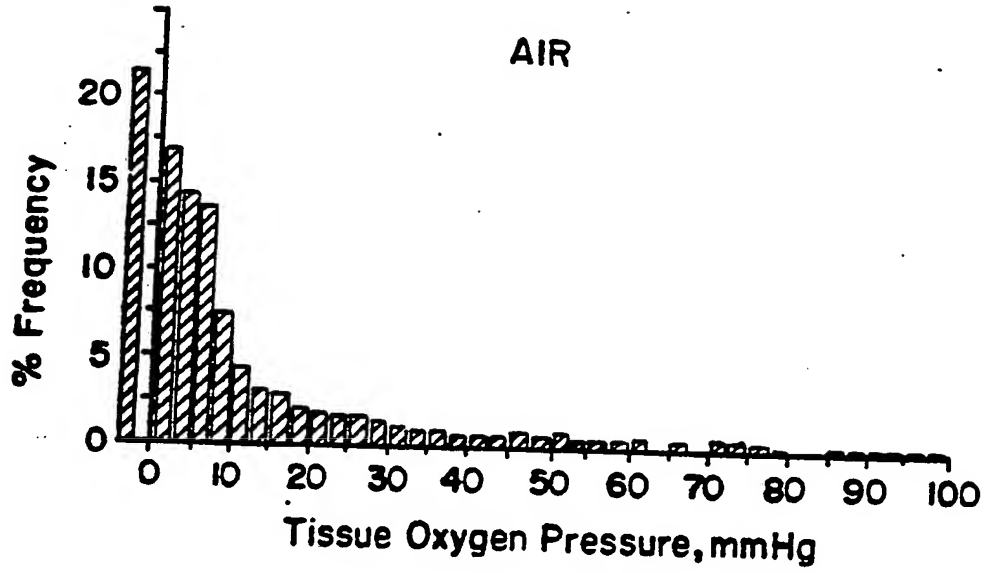
AIR
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3A

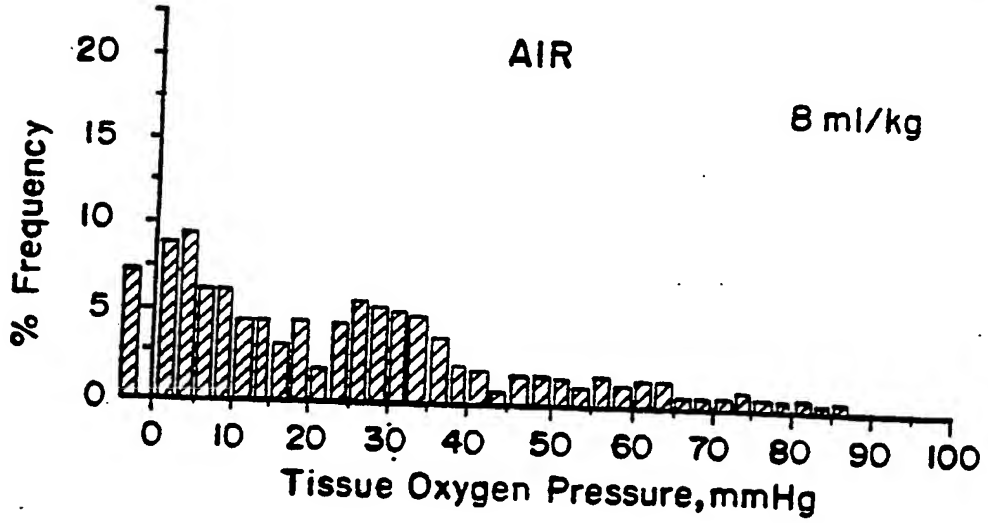
AIR
8 ml/kg
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3B

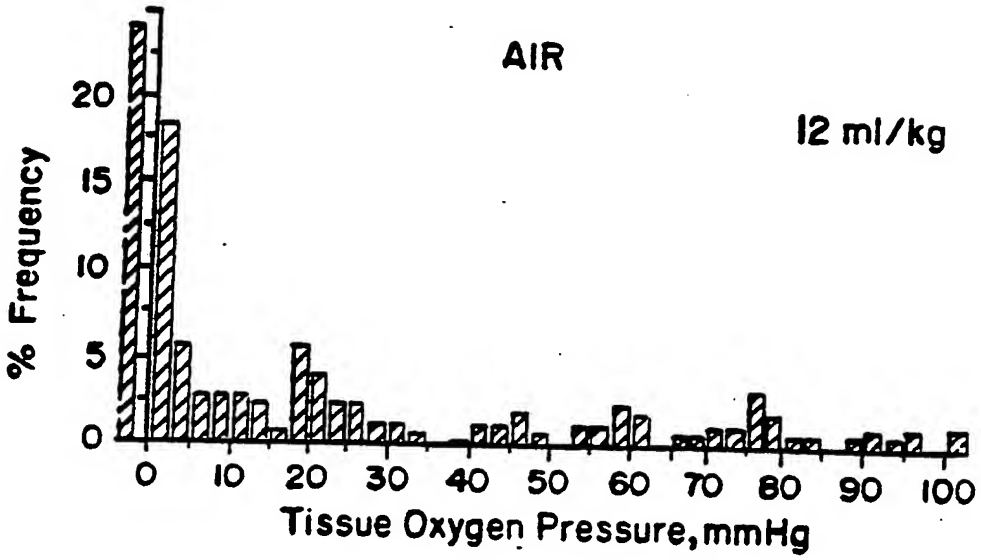
AIR
12 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3C

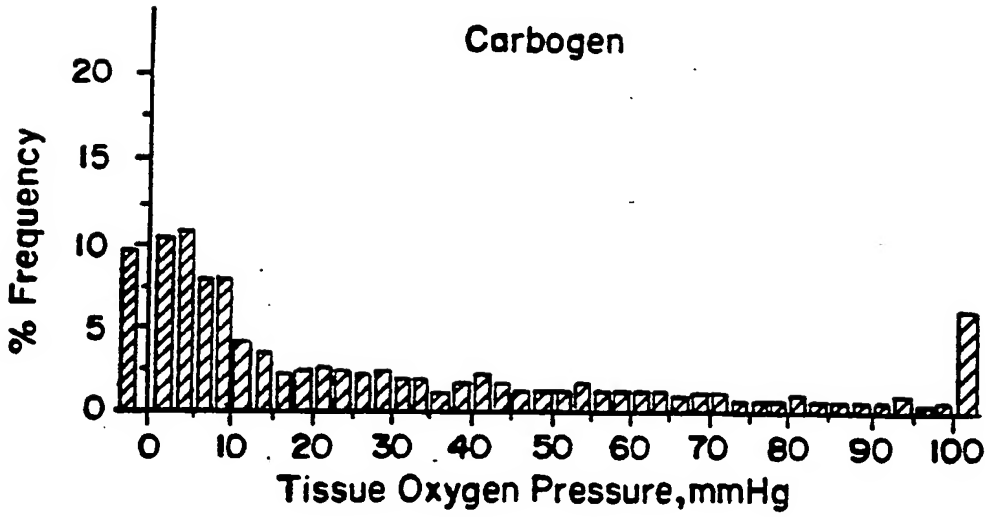
Carbogen
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3D

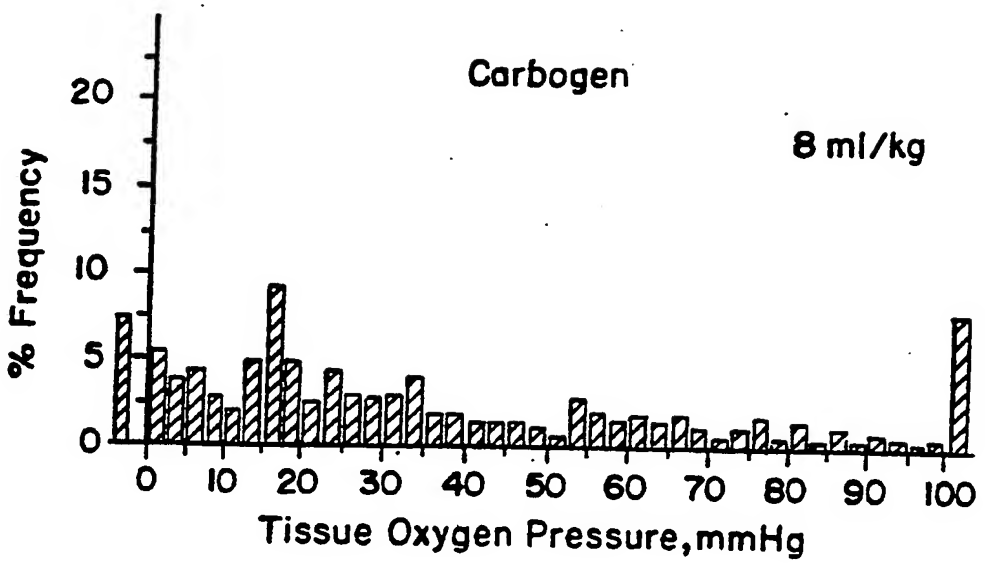
Carbogen
8 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3E

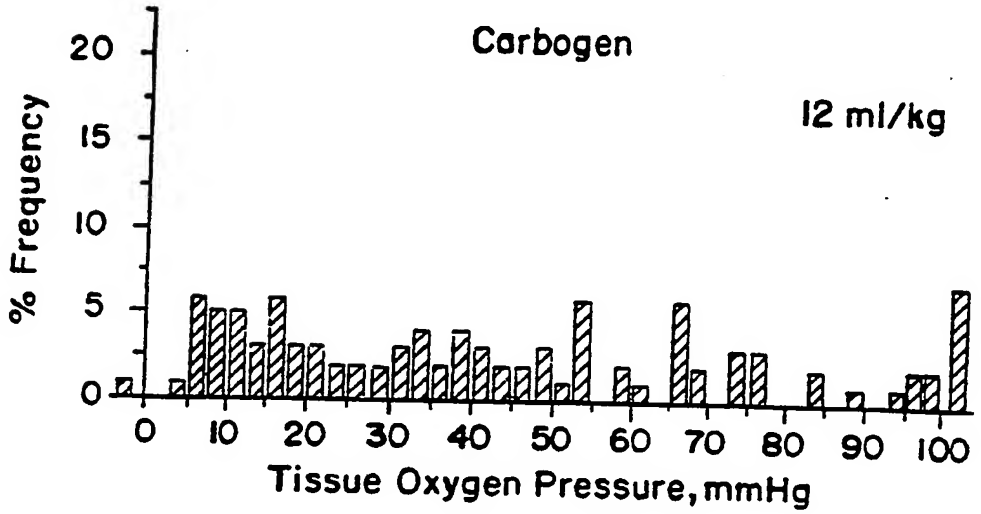
Carbogen
12 ml/kg
% Frequency
20
15
10
5
0
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 3F

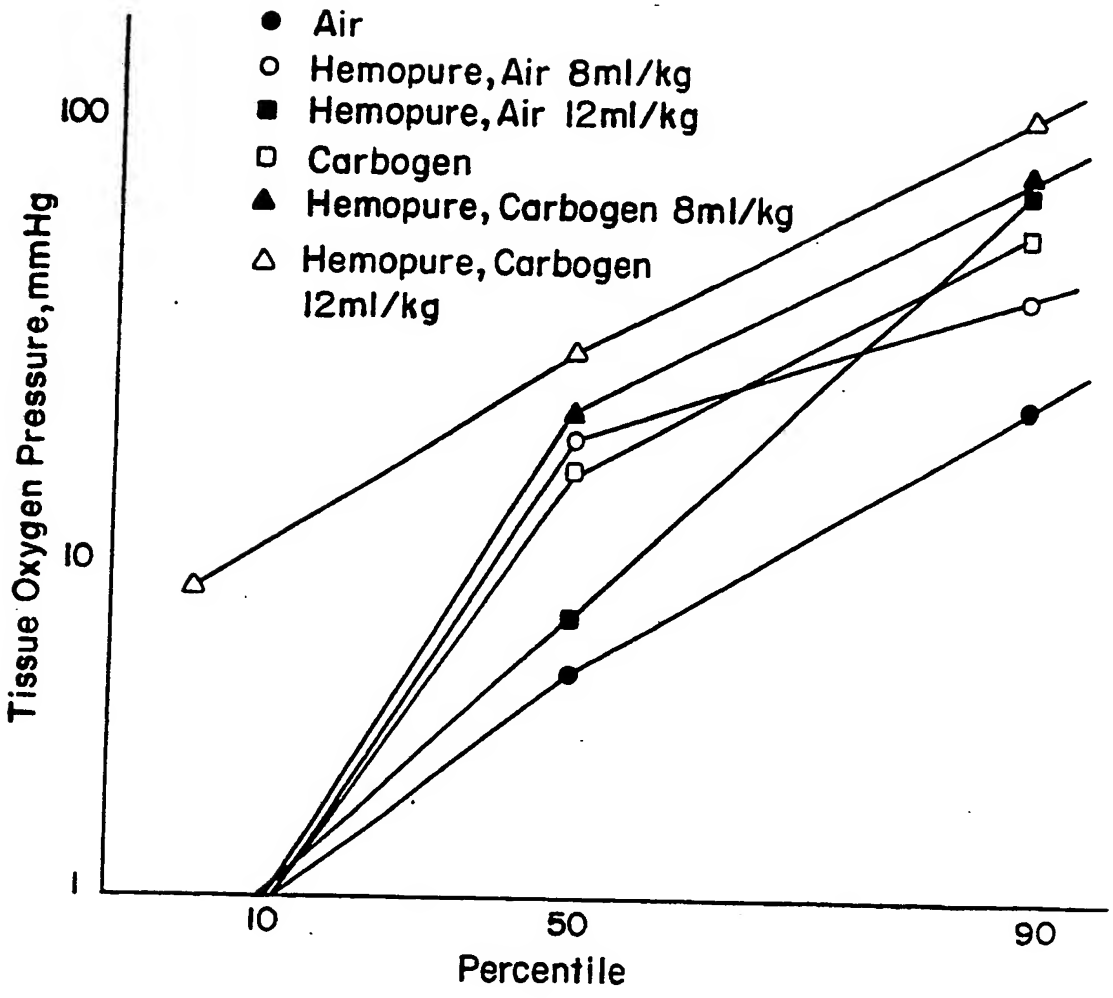
Air
Hemopure, Air 8ml/kg
Hemopure, Air 12ml/kg
Carbogen
Hemopure, Carbogen 8ml/kg
Hemopure, Carbogen 12ml/kg
Tissue Oxygen Pressure, mmHg
100
10
1
10
50
90
Percentile

FIG. 4

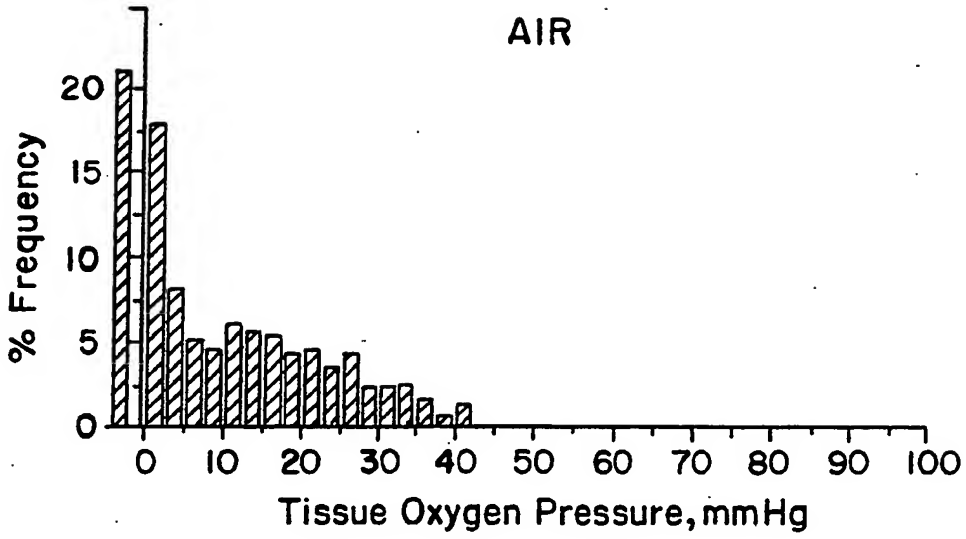
AIR
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5A

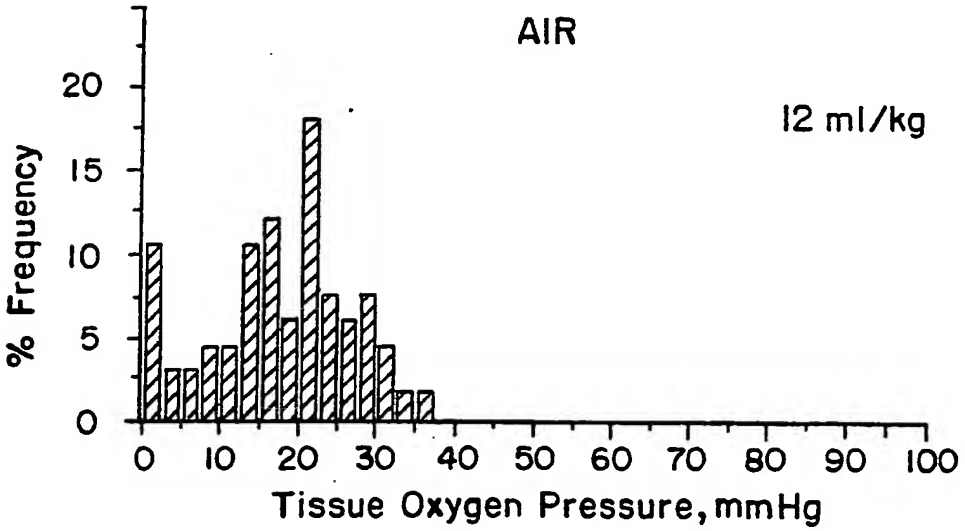
AIR
12 ml/kg
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5B

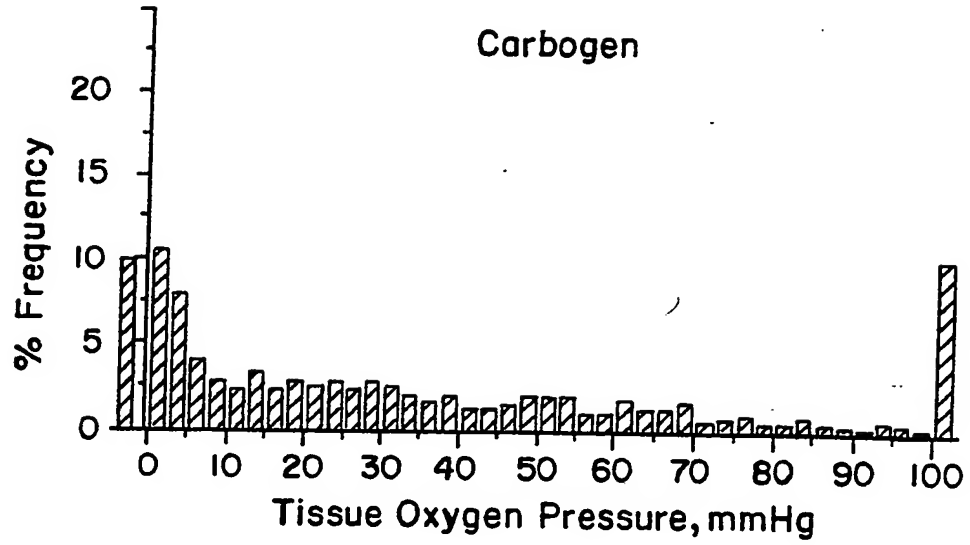
Carbogen
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5C

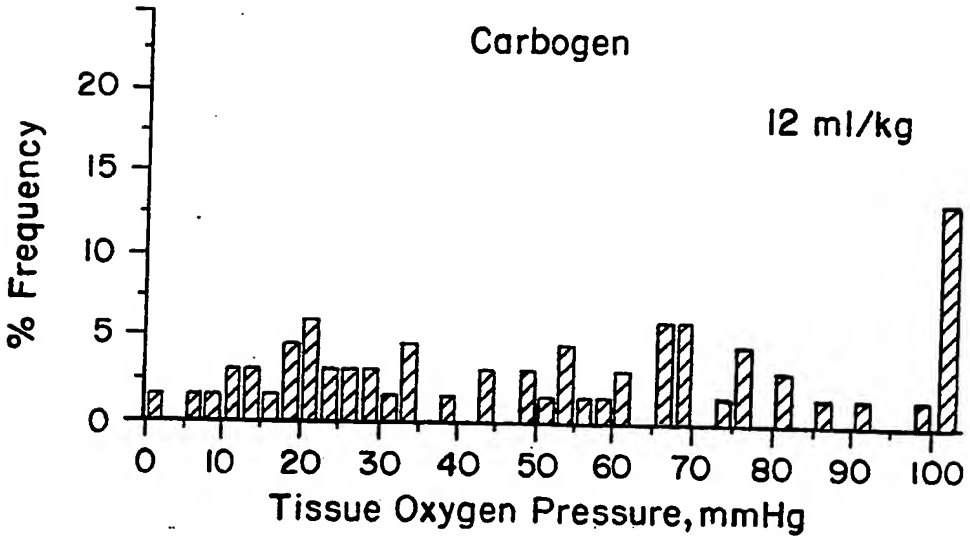
Carbogen
12 ml/kg
% Frequency
0
5
10
15
20
0 10 20 30 40 50 60 70 80 90 100
Tissue Oxygen Pressure, mmHg

FIG. 5D

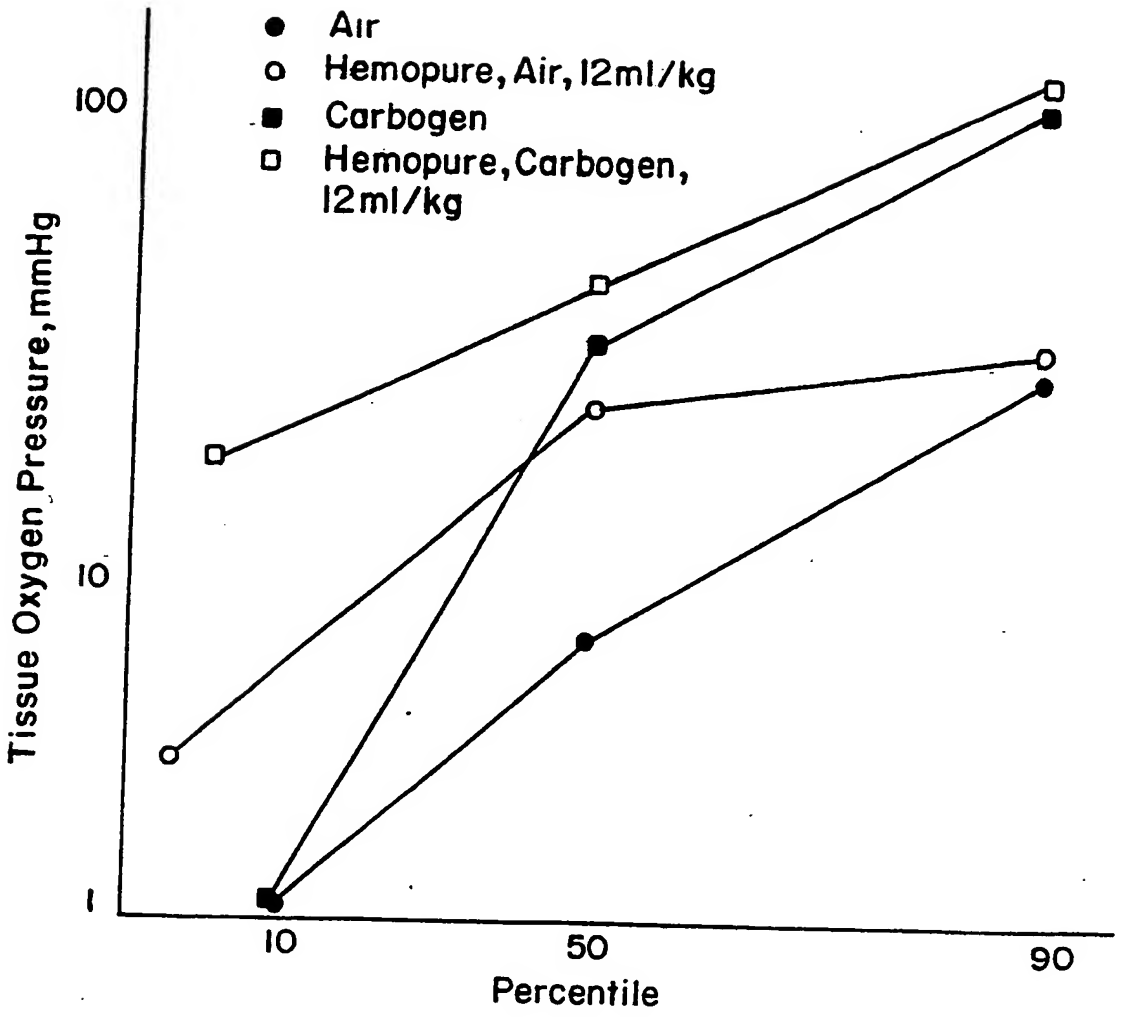
Air
Hemopure, Air, 12ml/kg
Carbogen
Hemopure, Carbogen, 12ml/kg
100
10
1
Tissue Oxygen Pressure, mmHg
10
50
90
Percentile

FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/04068

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 37/14 A 61 K 45/06

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Proceedings of the American Association for Cancer Research, vol. 32, March 1991, B.A. TEICHER et al.: "Combination treatment with a purified hemaglobin preparation and antitumor alkylating agents (AA) in the FSaIIC fibrosarcoma", page 387, abstract no. 2302, see abstract & 82 Ann. Meeting, Houston, 15-13 May 1991	1-9

-/-

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21-09-1992	29. 10. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT FFICE	Dagmar Frank
	Mme Dagmar FRANK

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	Biomaterials, Artificial Cells, and Immobilization Biotechnology, vol. 19, no. 2, 1991, B.A. TEICHER et al.: "Effect of a bovine hemoglobin preparation (PBHS) on the response of two murine solid tumors to radiation therapy and chemotherapeutic alkylating agents", page 491, see abstract & VIII World Congress of the International Society for Artificial Organs and the IV International Symposium on Blood Substitutes, Montreal, Quebec, 19-23 August 1991 ---	1-9
P,X	J. Cancer Res. Clin. Oncol., vol. 118, no. 2, February 1992, Springer-Verlag, B.A. TEICHER et al.: "Effect of a bovine hemoglobin preparation on the response of the FSaIIC fibrosarcoma to chemotherapeutic alkylating agents", pages 123-128, see the whole article ---	1-9
Y	Int. J. Radiat. Oncol. Biol. Phys., vol. 19, suppl. 1, 1990, B.A. TEICHER et al.: "Effect of oxygen level on the enhancement of tumor response to radiation by perfluorochemical emulsions (PFCES) or hemoglobin", page 137, abstract no. 27, see abstract & 32 Annual ASTRO Meeting, Miami Beach, 15-19 October 1990 ---	1-9
Y	STN International, File CA, Chemical Abstracts, vol. 101, no. 23, (Columbus, Ohio, US), see abstract no. 204312s, & JP,A,59130812 (GREEN CROSS CORP.) 27 July 1984, see abstract -----	1-9